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High-Yield Production of Secreted Active Proteins by the *Pseudomonas aeruginosa* Type III Secretion System▽†

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The *Escherichia coli* system is the system of choice for recombinant protein production because it is possible to obtain a high protein yield in inexpensive media. The accumulation of protein in an insoluble form in inclusion bodies remains a major disadvantage. Use of the *Pseudomonas aeruginosa* type III secretion system can avoid this problem, allowing the production of soluble secreted proteins.

Recombinant proteins are becoming increasingly valuable in different areas of research, including structural analysis studies, diagnostic investigation, and the treatment and prevention of various human diseases. Among the different expression systems commonly used for recombinant protein production in research and industry, the *Escherichia coli* system is often preferred if glycosylation and other posttranslational modifications are not required for the biological activity of the protein. The advantages of bacterial expression methods result from the ability of bacteria to reproduce rapidly to high densities on inexpensive substrates, their ease of manipulation, and the high protein yields that can be obtained in bacterial expression systems (11). Due to intracytosolic conditions not adapted for proper folding of polypeptide chains, however, some difficult-to-express proteins are often produced in *E. coli* in an undesirable insoluble form in inclusion bodies. To avoid the problem of insolubility and to facilitate purification, Mergulhão et al. have proposed use of a bacterial expression system with the intrinsic ability to secrete recombinant protein (11). Furthermore, protein secretion during bacterial growth could permit the development of a continuous process for recombinant protein production. However, the process of secretion in bacteria is complex, and the yields of secreted recombinant proteins are often low.

Gram-negative bacteria use different types of secretion systems for their own purposes. For example, the type III secretion system (TTSS) is involved in the cytotoxicity of several pathogenic bacteria (*Salmonella* spp., *Yersinia* spp., *Shigella* spp., *Pseudomonas* spp.) (1). The flagellar TTSS of *E. coli* has been previously shown to be a powerful tool for the production of recombinant proteins (10). The mechanisms of substrate recognition and secretion by TTSSs are complex for two reasons. First, it has been proposed that during the synthesis of the naturally secreted type III proteins, their correct folding is maintained by a specific interaction with a small acidic chaperone (2, 19). Second, once the proteins are secreted, they could be more easily folded with appropriate disulfide bonding, since the extracellular space is an oxidative environment (17). Although secretion signals seem to be localized in the N-terminal parts of proteins, these termini are not conserved between proteins secreted by the same TTSS (7). The *Pseudomonas aeruginosa* TTSS naturally delivers four types of large effector proteins (exotoxins S, T, Y, and U, with molecular masses of 49, 53, 42, and 74 kDa, respectively) into the cytoplasm of target cells in vivo. It has also been previously shown that direct TTSS secretion in the surrounding media occurs in vitro when *P. aeruginosa* is grown in calcium-depleted conditions (6). Taking advantage of these properties, we engineered an attenuated *P. aeruginosa* strain and a dedicated expression plasmid to secrete recombinant proteins into bacterial growth medium.

We previously showed that there were high levels of secretion when each of the following reporter proteins was fused with the 54 N-terminal amino acids of ExoS: *P. aeruginosa* inhibitor of vertebrate lysozyme (IVY), *Pseudomonas putida* catechol 2,3-dioxygenase, and green fluorescent protein (4). In the present study, we checked whether shorter tags could also allow the secretion of fusion proteins. As shown in Fig. 1A, the 17, 30, or 42 N-terminal amino acids of ExoS did not allow secretion of MRP8, a human soluble small protein with a molecular mass of 11 kDa belonging to the S100 family (Table 1). Considering the high homology of ExoT with ExoS, MRP8 was also fused to the 129, 96, 54, or 17 N-terminal amino acids of ExoT. The largest three constructs were secreted, while the small ExoT17-MRP8 construct was not (Fig. 1B), and the secretion levels were comparable to those seen for the ExoS42 fusion protein. Therefore, we considered the first 54 residues of ExoS the optimized N-terminal domain required for the secretion of fusion protein. We then designed and constructed the expression plasmid pEAI-S54 as follows. First, *exsA*, the gene coding for the common TTSS regulator of *P. aeruginosa*, was amplified by PCR using PfuUltra (Stratagene) with *P. aeruginosa* chromosomal DNA as the template and primers EXSAS and EXSAR (see Table S1 in the supplemental material). The PCR product was cloned as an EcoRI/HindIII fragment into EcoRI/HindIII-digested pTTQ18 (16) to pro-

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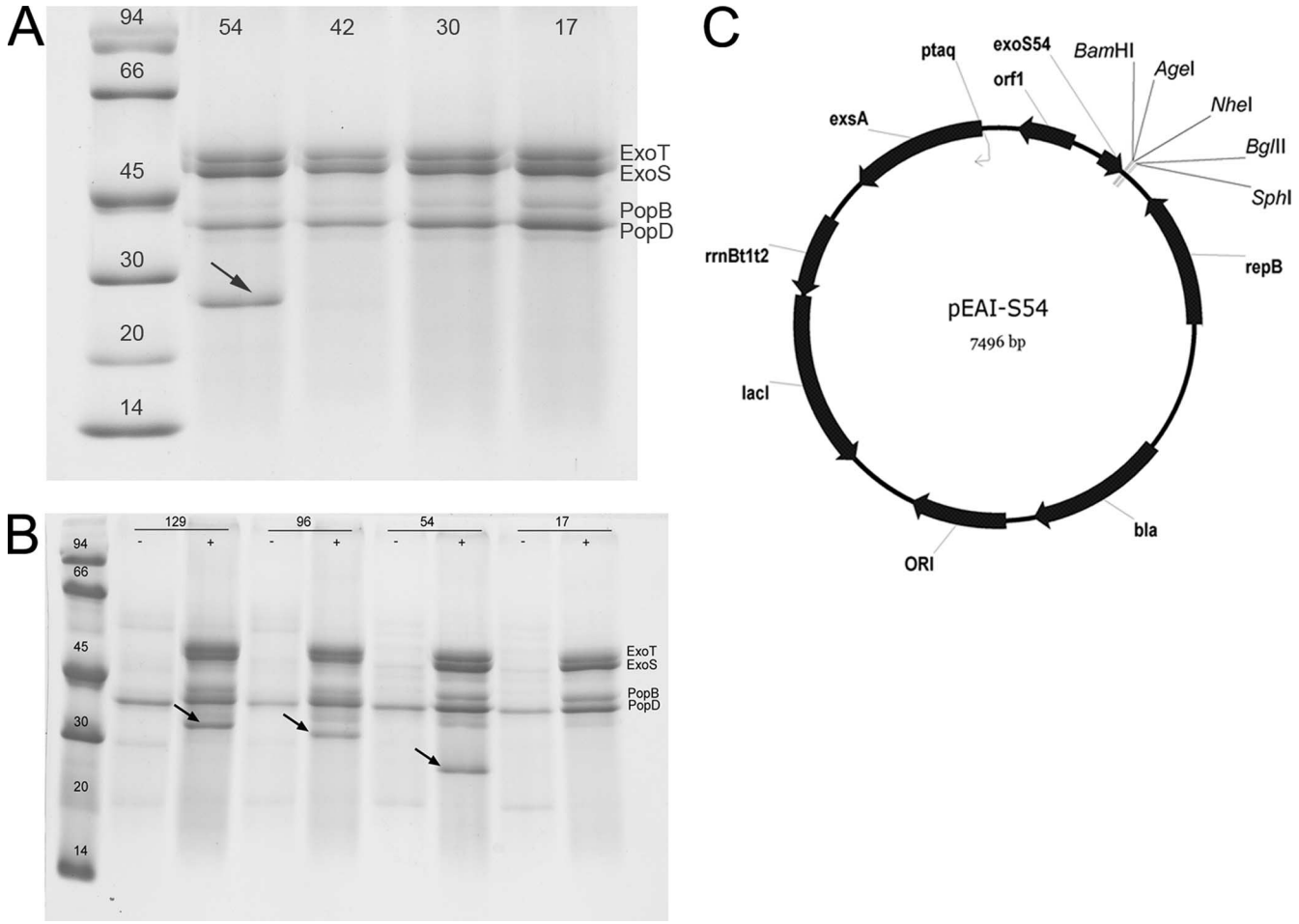

FIG. 1. (A) Secretion of MRP8 fused to the 54, 42, 30, and 17 N-terminal amino acids of ExoS. The *P. aeruginosa* CHA strain was transformed with the designed constructs by electroporation. A single colony from *Pseudomonas* isolation agar plates was subsequently inoculated into 1.5 ml Luria-Bertani medium supplemented with 300 $\mu g \cdot ml^{-1}$ carbenicillin and grown overnight at 37°C. After harvesting by centrifugation and a wash step, bacteria were resuspended in fresh Luria-Bertani medium to an optical density of 0.2. Protein expression was induced by addition of 0.5 mM IPTG, and protein secretion was induced by addition of 5 mM EGTA and 20 mM $MgCl_2$, followed by 3 h of incubation at 37°C. Cells were pelleted from 2 ml of a bacterial culture by centrifugation (15 min, 13,000 × *g* at room temperature). The clarified supernatant was precipitated with 12.5% trichloroacetic acid for 30 min on ice and centrifuged at 18,000 × *g* at 4°C, and the protein pellets were washed two times at −20°C with cold acetone and resuspended in electrophoresis buffer. Proteins were separated on a 12% sodium dodecyl sulfate-Tricine gel and visualized by Coomassie blue staining. ExoT (53 kDa) and ExoS (49 kDa) are TTSS toxins, and PopB (40 kDa) and PopD (31 kDa) are TTSS structural proteins secreted by the CHA strain. The position of the fusion protein is indicated by an arrow. (B) Secretion of MRP8 fused to the 54, 42, 30, and 17 N-terminal amino acids of ExoT. The experiment was performed as described above for the ExoS-MRP8 fusion. The position of the fusion protein is indicated by arrows. (C) Plasmid map of the pEAI-S54-EC-BS plasmid. pEAI-S54-EC is derived from the pUCP20 *P. aeruginosa* shuttle vector (GenBank accession no. U07165). Antibiotic resistance is encoded by *bla* (ampicillin). *ori* is the ColE1 origin of replication. The *lac* promoter (ptaq), *rrnb*T1T2 terminator fragment, and *lacI*^{q} repressor protein gene (lacI) are from pTTQ18 (GenBank accession no. CS410149). *repB* is the replication protein gene (Entrez protein no. AAB40009) from pUCP20. ExsA is the common TTSS regulator of *P. aeruginosa* (6); *orf1* encodes the specific ExoS chaperone for TTSS secretion (15); and *exoS54* is the sequence coding for the 54 first amino acids of the ExoS toxin that allow the secretion of the protein of interest by the TTSS of *P. aeruginosa*.

duce pTTQ18exsA. The expression of the *exsA* gene was under the control of the inducible promoter P*tac*. The whole inducible system, including the genes coding for the P*lac* repressor *lacI*^{q}, *exsA*, and the P*tac* promoter, was amplified from pTTQ18exsA with the Xbaptac and KpnlacIq primers (see Table S1 in the supplemental material) and cloned as an XbaI/KpnI fragment into pUCP20 (18) to produce pExaInd. Then the *orf1* gene and the fragment of the *exoS* gene coding for the 54 N-terminal amino acids of ExoS was amplified from *P. aeruginosa* chromosomal DNA with primers Orf1-Exos54S and Orf1-Exos54R and cloned as an XbaI/SphI fragment into XbaI/SphI-digested pExaInd. The resulting plasmid was designated pEAI-S54-BS and contains unique BamHI and SphI restriction sites. The final vector was obtained by insertion of the hybridized oligonucleotides LinkS and LinkR into BamHI/SphI-digested pEAI-S54-BS. The final cloning plasmid possessed a polylinker containing unique sites for BamHI, AgeI, NheI, BsrGI, BglI, and SphI and allowed easy cloning of genes coding for proteins of interest; this plasmid was designated pEAI-S54 (Fig. 1C). Plasmid pEAI-S54 is easily introduced into *P. aeruginosa* either by conjugation with *E. coli* strain S17.1 (see Table S1 in the supplemental material) or by

TABLE 1. Peptides and proteins secreted with the TTSS of *P. aeruginosa*[a]

Protein	Size (amino acids)	Molecular mass (kDa)	Secretion	Activity	Strain
gp100			++++	ND	CHA-OST
OVA	201	21.7	++++	ND	CHA-OST
CSP1	80	9.2	+++	ND	CHA-OST
GB1	115	12.6	+	ND	CHA-OST
GB2	90	9.1	++	ND	CHA-OST
IVY	189	21.3	+++	+	CHA
ExoS-MRP8 fusions					
ExoS54-MRP8	151	17.1	++	+	CHA
ExoS42-MRP8	139	15.8	−	ND	CHA
ExoS30-MRP8	127	14.5	−	ND	CHA
ExoS17-MRP8	114	13.2	−	ND	CHA
ExoT-MRP8 fusions					
ExoT129-MRP8	226	24.8	++	ND	CHA
ExoT96-MRP8	193	21.4	++	ND	CHA
ExoT54-MRP8	151	17.1	++	ND	CHA
ExoT17-MRP8	114	13.1	−	ND	CHA
$p67^{phox}$	657	73.7	+	+	CHA
XylE	439	49.1	±	+	CHA

[a] MRP8 was fused to various numbers of amino acids from the N-terminal end of ExoS or ExoT, as indicated. MRP8 and $p67^{phox}$ were tested using an NADPH oxidase activity test as described previously (13). XylE catechol dioxygenase activity was evaluated as described previously (5). *P. aeruginosa* IVY was evaluated on the basis of its ability to inhibit hen egg white lysozyme activity during lysis *Micrococcus lysodeikticus* cells, as described previously (12).

electroporation of plasmid DNA (3). This plasmid allows, with stimulation by isopropyl-β-D-thiogalactopyranoside (IPTG), activation of the whole TTSS. The synthesized protein of interest can then be secreted under calcium deprivation conditions, which are induced by addition of EGTA to the culture medium.

As shown in Fig. 1A and B, ExoS and ExoT, the secreted TTSS toxins of *P. aeruginosa*, represent the major protein contaminants. Therefore, to increase the purity and yield of recombinant proteins in the supernatant, we used the CHA-OST strain, which has deletions in the genes coding for these toxins, as previously described by Quénée et al. (14). Using this strain, different proteins were secreted using the approach described above. The results are summarized in Table 1. Among these proteins, all four proteins examined were shown to be truly active, suggesting that they were properly folded and disulfide bonded. To further increase the protein yield before purification, we developed a simple process to concentrate the recombinant protein in the supernatant. To avoid any contamination and facilitate downstream processing, the recombinant proteins were produced in chemically defined classical RPMI mammalian cell cultivation media. In the example given here, ExoS54 fused to the human gp100 protein was very efficiently secreted (Fig. 2).

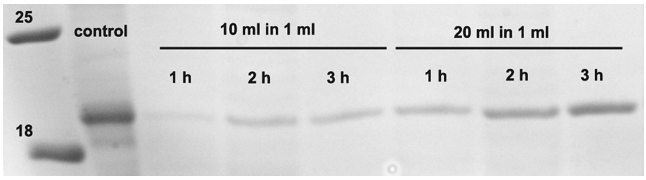

FIG. 2. Protein secretion with the CHA-OST strain in RPMI medium. Precultures (10 or 20 ml) were started in Luria-Bertani medium supplemented with IPTG. At an optical density at 600 nm of 1.8, bacteria were recovered, washed with RPMI medium, and concentrated in 1 ml of RPMI culture medium. Protein secretion was induced by EGTA. After 1, 2 or 3 h, 20 μl of culture medium was analyzed for protein secretion by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Coomassie blue staining. The control was 2 ml of culture supernatant precipitated and analyzed as described in the legend to Fig. 1.

In conclusion, we developed a completely novel expression system for recombinant protein production in *P. aeruginosa*, including a plasmid for inducible expression (pEAI-S54) and a bacterial strain that allows production and secretion of a wide variety of proteins in the active form. This system offers a new alternative solution to one of the main drawbacks of protein production with bacteria: the accumulation of protein as inclusion bodies in the bacterial cytosol. Although it has been demonstrated that coexpression of a chaperone (9) or fusion of the protein of interest with thioredoxin (8, 20) allows the production of recombinant protein in a soluble, active form in *E. coli*, the proteins are produced in the bacterial cytosol. Therefore, we propose that our system should be exploited to reduce insolubility problems, since it permits the targeted secretion of recombinant proteins through the TTSS of *P. aeruginosa*. We hypothesize that, due to the presence of dedicated intracellular chaperones acting on the ExoS54 sequence, the formation of inclusion bodies is avoided. In addition, the secretion of proteins into the oxidative extracellular medium could favor natural folding and disulfide bridge formation. Furthermore, the expression yield of the different recombinant proteins tested is sufficient to allow the development of straightforward purification schemes. We suggest that use of the bacterial TTSS constitutes an alternative to the eukaryotic cellular system for laboratory or industrial production of fully soluble recombinant proteins and their easy purification from inexpensive and well-defined culture media.

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